

Title

Dual-Targeted CAR- $\gamma\delta$ T Cells Overcome Antigen Heterogeneity in Metastatic Castration-Resistant Prostate Cancer

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Background

Metastatic castration-resistant prostate cancer (mCRPC) exhibits substantial spatiotemporal heterogeneity, with target expression varying across metastatic sites and evolving under therapeutic pressure, limiting the efficacy of single-antigen targeted therapies. Allogeneic CAR $\gamma\delta$ T cells are an attractive platform for therapy due to their intrinsic cytotoxicity and MHC-independent tumor recognition. Incorporation of dual-antigen targeting may further broaden anti-tumor function and reduce frequency of antigen escape. To achieve this, we developed dual-targeted, cytokine-armored CAR- $\gamma\delta$ T cells against prominent mCRPC antigens to enhance tumor recognition, sustain cytotoxicity, and overcome immunosuppression within the tumor microenvironment.

Methods

A panel of dual-targeting CARs recognizing STEAP1 and PSMA with differing construct architectures were generated and engineered into $\gamma\delta$ T cells to assess function. CD8 hinge-CD8TM or IgG4 hinge-CD28TM domains were incorporated into these constructs to promote receptor dimerization and enable shared signaling through a single CD3 ζ module. Single-target CARs incorporating either CD28-CD3 ζ or 4-1BB-CD3 ζ served as controls. Cytotoxicity assays were conducted to evaluate the anti-tumor activity of each CAR construct against tumor cells expressing both antigens, cells lacking either STEAP1 or PSMA, and heterogeneous mixtures of the two single-antigen-loss variants. Furthermore, the impact of IL-2 armoring was assessed using serial-killing and 3D spheroid-killing assays.

Results

Among the CAR designs evaluated, SP7 (STEAP1-BB ζ /PSMA-28 CAR) demonstrated the most effective tumor elimination despite antigen loss and was selected for further development. In mixed tumor cultures containing STEAP1- or PSMA-loss variants, single-target STEAP1 and PSMA CAR- $\gamma\delta$ T cells achieved maximal tumor-cell killing of 59% and 69%, respectively, at 48 hours, followed by cancer cell regrowth. In contrast, SP7 eliminated 83% of the heterogeneous cancer cell population at 48 hours and 93% at 120 hours, demonstrating sustained cytotoxicity and broad coverage of heterogeneous cancer cell population. In serial-killing assays against STEAP1+ PSMA+ 22Rv1, SP7 retained 57% killing in second round challenges, compared with 24% and 2% for STEAP1- and PSMA-targeted CAR- $\gamma\delta$ T cells, respectively. Additionally, armoring of STEAP1-BB ζ /PSMA-28 CAR- $\gamma\delta$ T cells with IL-2 extended cytotoxic activity through three rounds of tumor cell challenge, whereas non-armored controls failed to achieve complete cancer cell clearance during the second challenge and exhibited significantly higher TIGIT and TIM-3 expression. In

three-dimensional spheroid assays, IL-2-armored, but not control, CAR- $\gamma\delta$ T cells (E:T ratio 2.5), cleared tumor spheroids within 7 days co-culture.

Conclusions

The dual-targeted STEAP1-BB ζ /PSMA-28 CAR maintained cytotoxicity against heterogeneous cancer cell populations despite loss of either target antigen and showed more sustained activity than the corresponding single-target CARs. IL-2 armoring further enhanced cytotoxic function during repeated cancer cell challenges and afforded tumor eradication in three-dimensional spheroid models, supporting further evaluation of IL-2-armored SP7 in preclinical *in vivo* models of antigen-heterogeneous mCRPC.

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Conflicts of Interest Disclosure Statement

BRW and BM are founders and hold equity in Luminary Therapeutics.